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Enzyme Labeling of Steroids by the Activated Ester Method[1)]

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Enzyme labeling of a steroid by the activated ester method has been investigated. The reaction of the N-hydroxysuccinimide ester of a testosterone derivative with β-D-galactosidase provided a conjugate suitable for enzyme immunoassay. Dose-response curves with satisfactory sensitivity and range of measurement could be obtained by the use of conjugates prepared at a limited molar ratio of steroid to enzyme. The activated ester method proved to be useful for enzyme labeling of antigen because of its simplicity and excellent reproducibility.

Keywords——enzyme immunoassay; enzyme labeling of steroid; activated ester method; N-hydroxysuccinimide ester; testosterone-β-D-galactosidase conjugate; double antibody method; molar ratio of steroid to enzyme; immunoreactivity; sensitivity; dose-response curve

Enzyme-labeled antigens for use in the enzyme immunoassay of steroid hormones have usually been prepared by condensation of the carboxyl group of a steroid with the amino group of a lysine residue in an enzyme. The sensitivity and reproducibility of enzyme immunoassays are influenced by the coupling method; minimum loss of immunoreactivity and enzymic activity are essential. However, the commonly used techniques, *i.e.*, the mixed anhydride and carbodiimide methods, are not always satisfactory. In our previous paper we reported the synthesis of N-hydroxysuccinimide esters of steroids and their reactivities with bovine serum albumin (BSA).[3)] This paper deals with the preparation of testosterone-β-D-galactosidase (EC 3.2.1.23) conjugates by the activated ester method.

Materials and Methods

Reagents——β-D-Galactosidase from *E. coli* (grade VI, 290 Units per mg protein) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and *o*-nitrophenyl-β-D-galactopyranoside from Nakarai Chemicals, Ltd. (Kyoto). The N-hydroxysuccinimide ester of 4-hydroxytestosterone 4-hemiglutarate[4)] was prepared by the method previously established in these laboratories.[3)] Anti-testosterone antiserum was produced by immunization using the conjugate of 4-hydroxytestosterone 4-hemiglutarate with BSA in the rabbit, as reported in the previous paper.[4)] The serum was diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (buffer A). Goat anti-rabbit IgG antiserum and normal rabbit serum were kindly provided by Eiken Kagaku Co., Ltd. (Tokyo).

Preparation of Testosterone-β-D-Galactosidase Conjugate——Method I: A solution of the N-hydroxysuccinimide ester (9.3—111 nmol) in dioxane (0.1 ml) was added to a stirred solution of β-D-galactosidase (1 mg) in PB (0.5 ml) at 0°, and the mixture was gently stirred at 4° for 2 hr. The resulting solution was dialyzed against cold PB (1 liter) for 2 days.

Method II: A solution of the N-hydroxysuccinimide ester in CH_2Cl_2 (1.85×10^{-3} M) was transferred to a test tube in 1—10 μl aliquots using a micro syringe, and the solvent was evaporated off. A solution of β-D-galactosidase (1 mg) in PB (0.2 ml) was added to the residue at 0°, and the reaction mixture was immediately vortex-mixed, then allowed to stand overnight at 4° with occasional shaking.

1) Part CIL of "Studies on Steroids" by T. Nambara; Part CXXXXVIII: K. Shimada and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **27**, 1881 (1979).
2) Location: *Aobayama, Sendai, 980, Japan*; *a*) To whom inquiries should be addressed.
3) H. Hosoda, Y. Sakai, H. Yoshida, S. Miyairi, K. Ishii, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **27**, 242 (1979).
4) H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, **10**, 513 (1979).

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The solutions were stored at 4° at a concentration of 200 μg per ml, adjusted with PB containing 0.1% gelatin and 0.1% NaN_3 (buffer B). For the immunoassay procedure, this was diluted with the buffer solution containing 0.5% normal rabbit serum.

Competitive Binding of Testosterone and the Testosterone-β-D-galactosidase Conjugate——Diluted antiserum (0.1 ml) and the testosterone-enzyme conjugate (0.1 μg, 0.1 ml) in the buffer were added to a series of standard solutions of testosterone (0—10 ng) in buffer B (0.1 ml), and the mixture was incubated at 4° for 4 hr. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1:30 with buffer A containing 0.3% ethylenediaminetetraacetic acid (EDTA) was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4° for 16 hr. After addition of buffer B (1 ml) the resulting mixture was centrifuged at 3000 rpm for 15 min. The supernatant was removed and the immune precipitate was used for measurement of the enzymic activity.

Measurement of β-D-Galactosidase Activity——The immune precipitate was diluted with buffer A (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37° for 3 min. *o*-Nitrophenyl-β-D-galactopyranoside (0.06%, 1 ml) in PB was added to the resulting solution and the mixture was incubated for 30—120 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). The absorbance was then measured at 420 nm with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

To test the recovery of enzymic activity in the coupling by Method I and II, conjugates corresponding to 0.1 μg of the enzyme were used, with β-D-galactosidase in PB stored at 4° at the same concentration as a control.

Results and Discussion

The N-hydroxysuccinimide ester prepared from 4-hydroxytestosterone 4-hemiglutarate by condensation with N-hydroxysuccinimide in the presence of a water-soluble carbodiimide[3] was covalently linked to β-D-galactosidase (Fig. 1). β-D-Galactosidase was chosen as a model labeling enzyme since it has been widely used for enzyme immunoassays of steroids. The enzyme labeling occurred readily, simply on mixing the activated ester with the enzyme in phosphate buffer either with or without dioxane. The activated ester should react readily with free amino groups and possibly with thiol groups of the enzyme.[5] Various molar ratios of the testosterone derivative to enzyme, ranging from 5 to 60 in Method I and from 1 to 10 in Method II, were used for coupling. The number of steroid molecules incorporated per enzyme molecule was not determined. The loss of the enzymic activity was less than 20% under the coupling conditions used.

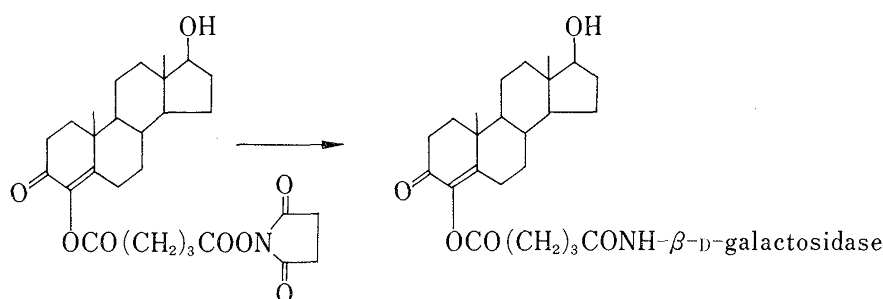

Fig. 1. Preparation of Testosterone-β-D-galactosidase Conjugate

The anti-testosterone antiserum used in the enzyme immunoassay was that raised against the conjugate of 4-hydroxytestosterone 4-hemiglutarate with BSA, that is, a homologous combination. The bound and free enzyme-steroid conjugates were separated by a double-antibody method, and the enzymic activity of immune precipitate was determined colorimetrically using *o*-nitrophenyl-β-D-galactopyranoside as a substrate.

The binding abilities of the enzyme-steroid conjugates obtained by Method I with various molar ratios of the activated ester to enzyme were investigated at 1:500 dilution of anti-testosterone antiserum (Fig. 2). The immunoreactivity increased with increasing molar ratio

5) P. Cuatrecases and I. Parikh, *Biochemistry*, 11, 2291 (1972).

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Table I. Inhibition of Bound Enzymic Activity by 1 ng of Testosterone

Molar ratio (steroid/enzyme)	Inhibition (%)
15	76
30	43
60	27

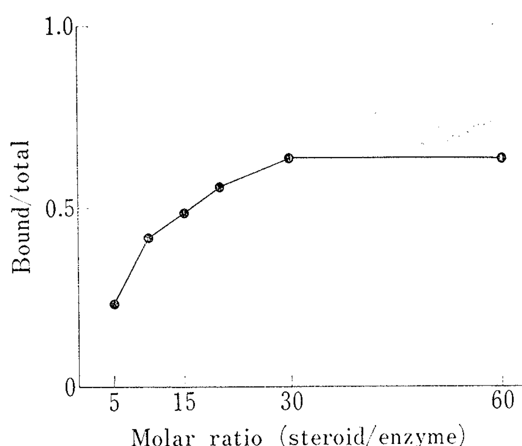

Fig. 2. Binding Abilities of Testosterone-β-D-galactosidase Conjugates prepared at various Molar Ratios of the Activated Ester to Enzyme

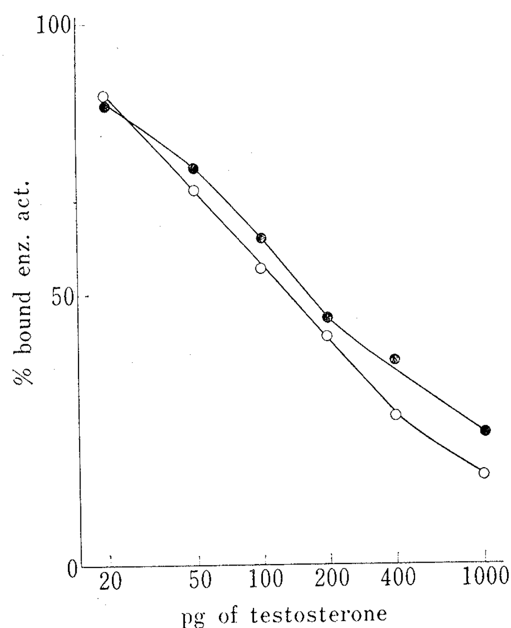

Fig. 3. Dose-Response Curves for Enzyme Immunoassay of Testosterone with Testosterone-β-D-galactosidase Conjugates prepared by Method I (-●-●-) and Method II (-○-○-)

Dilutions of anti-testosterone antiserum used were 1: 10000 and 1: 4000, respectively.

and reached a plateau with a ratio of 30. An immunoassay system requires efficient inhibition of enzymic activity on addition of antigen and an adequate optical density for B_0 during a limited incubation period. Therefore, the enzyme-steroid conjugates obtained were assessed in terms of an optical density of at least 0.2 after incubation for 2 hr with 1: 2000 dilution of antiserum. The results for inhibition of bound enzymic activity by the addition of 1 ng of testosterone per test tube at 1: 10000 dilution of antiserum are listed in Table I. Sufficient inhibition was observed at a molar ratio of 15, whereas a satisfactory result could not be obtained at a molar ratio of 60 even when the dilution of antiserum and the amount of enzyme-steroid conjugate were varied. This shows that the sensitivity is markedly influenced by the number of steroid molecules incorporated into the enzyme molecule.

An alternative coupling method (Method II) which eliminates the use of dioxane during coupling and dialysis was also developed. Inspection of the dose-response curves with the conjugates prepared by this method revealed that molar ratios ranging from 2 to 5 gave satisfactory results with respect to sensitivity and range of measurement (50—1000 pg). Typical dose-response curves with the conjugates prepared at molar ratios of 15 in Method I and 5 in Method II are shown in Fig. 3. The detection limit of testosterone in both cases was approximately 20 pg, comparable to those obtainable by enzyme immunoassay[6,7] and

6) K. Tateishi, H. Yamamoto, T. Ogihara, and C. Hayashi, *Steroids*, **30**, 25 (1977).
7) K.M. Rajkowski, N. Cittanova, P. Urios, and M.F. Jayle, *Steroids*, **30**, 129 (1977).

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radioimmunoassay. The steroid-enzyme conjugates prepared by these methods were found to be stable for several months as regards enzymic activity when stored at 4°.

It is noteworthy that satisfactory sensitivity can be obtained using enzyme-steroid conjugate which has not been purified by dialysis. However, the presence of carboxylated steroid contamination due to the elimination of dialysis would affect the assay sensitivity. If the profitable nature of affinity can be selected by employing "bridge" or "site" heterology, the sensitivity of assay may possibly be improved.

To the best of our knowledge this is the first reported enzyme labeling of steroids by the activated ester method. The present method may be applicable to other enzymes; it offers simplicity and excellent reproducibility for enzyme immunoassay of steroid hormones and drugs. The coupling reaction can be accomplished in various buffer solutions (pH 5—10) without any nucleophilic reagents capable of reacting with the activated ester.[5] It has been found that the reaction of N-hydroxysuccinimide esters of other carboxylated testosterones[3] with the enzyme also provides immunoreactive conjugates. The effect of heterologous combination between antiserum and enzyme-labeled testosterone on the sensitivity and specificity of enzyme immunoassay will be the subject of a future communication.

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